Karolinska Institutet

Department of Women´s and Children´s Health

Inflammation, caffeine and adenosine in neonatal hypoxic ischemic brain injury

AKADEMISK AVHANDLING
som för avläggande av medicine doktorsexamen vid Karolinska Institutet offentligen försvaras i Lars Leksells Auditorium T3 02 på Karolinska Universitetssjukhuset Solna

Fredagen den 28 februari, 2014, kl 09.00.

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Stockholm 2014

From Women´s and Children´s Health, Karolinska Institutet, Stockholm, Sweden

INFLAMMATION, CAFFEINE AND ADENOSINE IN NEONATAL HYPOXIC ISCHEMIC BRAIN INJURY

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Stockholm 2014

ISBN 978-91-7549-454-8

Printed by
REPROPRINT AB
Stockholm 2014
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

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Astrid Lindgren

1 ABSTRACT

Background: Brain injury during the neonatal period has potentially lifelong consequences for a child. Perinatal infections and inflammation can induce preterm birth and unfavorable cognitive development, Thus inflammation has received enthusiastic interest for potential therapeutic approaches seeking to protect the newborn brain. Experimental evidence demonstrates that inflammation induces brain injury succeeding the initial insult. A key cytokine in brain injury is the tumor necrosis factor (TNF-α), with reported detrimental cytotoxic effects on selected neuronal populations. Nonetheless, important functions of TNF-α in cerebral homeostasis and development have also been described. Caffeine is used against apneas of prematurity, with noticeable protection against cognitive delay and cerebral palsy. The main effects of caffeine at clinically relevant doses are mediated through inhibition of adenosine receptors. Adenosine is formed from adenosine triphosphate, the main transporter of chemical energy in the cell, which is readily cleaved to adenosine upon extracellular release or extensive leakage from injured necrotic cells. Hence, adenosine signaling is tightly interrelated with local energy levels and cell injury. In addition, adenosine modulates inflammatory responses in profound ways.

Methods: In mouse models of premature excitotoxic lesions and full term hypoxic ischemic brain injury we investigated blockade of TNF-α with and without interleukin-1 (IL-1) or lipopolysaccharide (LPS) induced systemic inflammation. Furthermore, in the hypoxic ischemic model we developed a flow cytometry based method to investigate temporal distribution of brain infiltrating and splenetic immune cells and their activation. To analyze the data in an unbiased way, we next adapted a data driven gating methodology. Moreover, we used principal component analysis to discriminate between experimentally entangled variables. Utilizing these techniques, we explored the effect of genetic inactivation of adenosine A_1 and A_{2A} receptors in the hypoxic ischemic model. We also tested the unselective, competitive adenosine receptor antagonist caffeine and assessed the effect on outcome and immune activation.

Results: Blockade of TNF-α protected the brain against excitotoxic lesions in the presence but not absence of systemic inflammation. No protection was observed in the full term hypoxic ischemic model. Persistent lymphocyte activation was found three months after the lesion. Moreover, spleenocytes harvested five months after neonatal brain damage proliferated when stimulated with brain homogenate in contrast to sham operated counterparts. Adenosine A_1 receptor deficient mice acquired significantly larger infarcts and associated adverse behavioral outcome compared to wild type. There were specific alterations in the immune responses induced after brain injury, including impaired cytotoxic function and dysregulation of regulatory B-lymphocytes. Adenosine A_{2A} receptor knockout mice developed increased atrophy compared to wild type after hypoxic ischemia, an effect accompanied by functional deficits in behavioral tests. Furthermore, a compensated functional insufficiency was estimated in the regulatory T-lymphocyte compartment in combination with a seemingly inadequate number of myeloid derived suppressor like cells, accompanied by a reversed, increased response in innate antigen presenting cells in the knockout. Finally, we report neuroprotective properties of 5 mg/kg caffeine given directly after neonatal brain injury.

Discussion: TNF-α blockade could potentially protect against preterm excitotoxic brain injury. Only patients with concurrent systemic inflammation would potentially benefit. Moreover, concern about adverse effects exists, why TNF-α blockade for neonatal brain injury is likely not clinically applicable in the near future.

Persistent long term cerebral adaptive immune activation, preceded by systemic immune activation in spleen was discovered. Remarkably, spleenocytes from animals subjected to brain injury responded to brain antigen five months after brain damage, whereas spleenocytes from uninjured did not, suggesting formation of immunological memory that might affect long term outcome and provoke autoimmunity later in life.

To avoid bias from manual gating of flow cytometry data we developed a data driven approach adapted for brain infiltrating immune cells. Furthermore, we deployed principal component analysis to verify biological relevance in the pattern of immune activation and to discriminate between genotype and injury size effects, since they are experimentally inseparable. Thus we could predict genotype and whether they acquired brain injury or not, from the flow cytometric immune activation pattern alone.

Adenosine A_1 receptor deficient mice display signs of regulatory B-lymphocyte dysfunction that imply a novel adenosinergic mechanism of B-lymphocyte regulation. In addition, these animals displayed signs of altered cellular cytotoxic immunity. Thus considerable effects on immune activation were present in the A_1 receptor knockouts compared to wild type, adding another mechanism linked to worse outcome after hypoxic ischemic brain injury in these animals.

Deletion of the adenosine A_{2A} receptor similarly causes worse outcome, however, the alteration of the immune response is completely different. Fundamental changes were observed in regulatory populations like monocyte derived suppressor like cells and regulatory T-lymphocytes. Extensive activation of cytotoxic populations in the adenosine A_{2A} receptor knockout links insufficient regulatory immune function with adverse behavioral and morphological outcome.

We also propose a novel hypothesis that short term blockade of adenosine A_{2A} receptors offers neuroprotection whereas long term blockade is detrimental by immunological mechanisms. Thus we tested the tentative therapeutic potential of caffeine, an unselective competitive antagonist of adenosine receptors. Caffeine 5mg/kg given directly after the insult resulted in reduced injury size after neonatal hypoxic ischemia. Since caffeine is a relatively well studied substance with negligible adverse long term effect in technically sound studies absent of significant bias, this approach has a clear clinical relevance. Are we ready for a clinical trial?

2 LIST OF PUBLICATIONS

Aden U, Favrais G, Plaisant F, **Winerdal M**, Felderhoff-Mueser U, Lampa J, et al. Systemic inflammation sensitizes the neonatal brain to excitotoxicity through a pro-/anti-inflammatory imbalance: key role of TNFalpha pathway and protection by etanercept. Brain Behav Immun. 2010 Jul;24(5):747-58.

Winerdal M, Winerdal ME, Kinn J, Urmaliya V, Winqvist O, Aden U. Long lasting local and systemic inflammation after cerebral hypoxic ischemia in newborn mice. PLoS One. 2012;7(5):e36422. doi: 10.1371/journal.pone.0036422PONE-D-11-17042 [pii]

Winerdal M, Winerdal ME, Fredholm BB, Winqvist O, Aden U. Adenosine A_1 Receptors Contribute to Immune Regulation after Neonatal Hypoxic Ischemic Brain Injury. *In manuscript.*

Winerdal M, Winerdal ME, Urmaliya V, Fredholm BB, Winqvist O, Aden U. Adenosine A_{2A} receptor deficiency increases hypoxic ischemic brain injury whereas the nonselective antagonist caffeine offers neuroprotection. *In manuscript.*

LIST OF PUBLICATIONS NOT INCLUDED IN THIS THESIS

Winerdal ME, Marits P, **Winerdal M**, Hasan M, Rosenblatt R, Tolf A, et al. FOXP3 and survival in urinary bladder cancer. BJU Int. 2011 Jan 18.

3 CONTENTS

4 LIST OF ABBREVIATIONS

A_1	Adenosine A_1 Receptors
A_{2A}	Adenosine A_{2A} Receptors
A_{2B}	Adenosine A_{2B} receptors
A_3	Adenosine A_3 Receptors
ADA	Adenosine Deaminase
ADK	Adenosine Kinase
APC	Antigen Presenting Cell
B10	Regulatory B-cells (producing IL-10)
DAMP	Damage Associated Molecular Pattern
DNA	DeoxyriboNucleic Acid
DNA	Deoxyribonucleic acid
EM	Expectation Maximization
FACS	Fluorescence Activated Cell Sorting
HI	Hypoxic Ischemia
IL	Interleukin
MCAO	Middle Cerebral Artery Occlusion
MDSC	Myeloid Derived Suppressor Cells
MHC	Major Histocompatibility Complex
NFκB	Nuclear factor κβ
PCA	Principal Component Analysis
RNA	Ribonucleic acid
SCID	Severe Combined Immuno Deficiency
TNF	Tumor Necrosis Factor
TNFR	Tumor Necrosis Factor Receptor
T-reg	Regulatory T-lymphocyte

5 BACKGROUND

Brain injury in the newborn child is a tragedy with potentially lifelong consequences. Injury to the developing brain inflicts disturbance not only to neuronal function, but growth and organization of the immature brain is impaired as well. Hence, developmental age is essential to the brain pathology seen (Inder and Volpe 2000). Premature children born during week 24-32 are predisposed to acquire white matter damage, since pre-oligodendrocytes mature to myelinate the neurons during this developmental period (Hagberg, Peebles et al. 2002). Immature oligodendrocytes are particularly susceptible to the reactive oxygen species formation that occurs during ischemia, whereas mature oligodendrocytes are more resistant to oxidative stress (Ferriero 2004). Periventricular hemorrhage provoking venous congestion and subsequent ischemia (Inder and Volpe 2000) is also quite common in premature babies, possibly due to immature vascular beds. Maternal or fetal inflammation is associated with preterm delivery that in itself hamper brain development and it has been proposed that infection could even cause direct cognitive deterioration (Dammann, Kuban et al. 2002). A current meta-analysis of neurodevelopmental outcome now conclude that perinatal infections influence outcome adversely (van Vliet, de Kieviet et al. 2013). Thus, inflammatory mediators are implicated in the pathogenesis of preterm brain injury, an hypothesis supported by accumulating human and experimental data (Volpe 2001).

However, in full term neonates selected populations of neurons are the cells most susceptible to harm, typically resulting in grey matter injury (Ferriero 2004). Asphyxia induced hypoxic ischemia (HI) is the major cause of neonatal brain injury in full term babies and the incidence of moderate to severe hypoxic ischemic encephalophathy is 0.5–2 per 1000 live births in the developed world (Kurinczuk, White-Koning et al. 2010) and much more common in low income countries (Costello and Manandhar 1994). There is hope though, since many neonates with brain injury have a brain function similar to uninjured children, illustrating the inherited inspiring regenerative capacity and plasticity of the neonatal brain (Ballantyne, Spilkin et al. 2008).

Furthermore, prognosis and survival after neonatal brain injury has improved due to better postnatal care with moderate cooling as the only specific treatment for neonatal hypoxic ischemic encephalopathy (Jacobs, Hunt et al. 2007). Major improvements have been achieved through the development of neonatal intensive care, with enhanced treatment of respiratory distress, sepsis, chock and use of drugs specifically adapted for neonates (Ferriero 2004). Improved obstetric care, nutrition, special consideration for premature infants and continuous support during the entire childhood are additional positive influencing factors (Luciana 2003).

5.1 INFLAMMATION

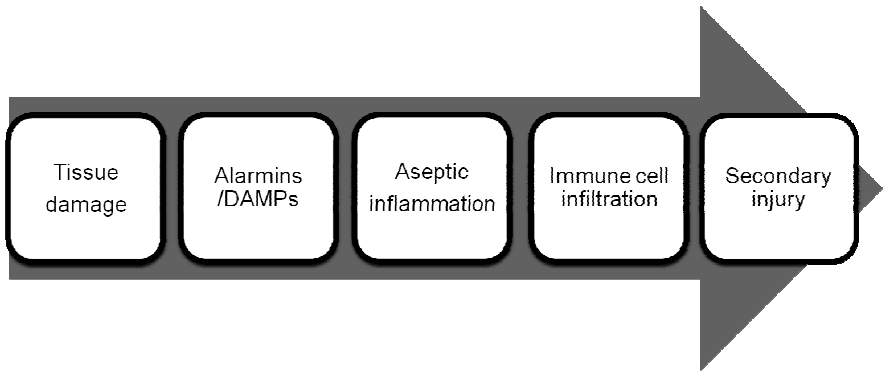

Figure 1 Tissue damage and disruption of cellular integrity releases intracellular molecules to the microenvironment that act as danger signals, which trigger an immune response to combat destructing agents. Some of the released substances have immunogenic properties and are named alarmins or damage associated molecular patterns (DAMPs). If no infection is present, they initiate an aseptic inflammation after brain injury, breaching the blood brain barrier for immune infiltration and secondary injury. When one contemplates this model, it is comprehensible that addition of an infectious or inflammatory agent can aggravate the injury.

Inflammation has been identified as a key factor for determining neurological outcome after asphyxia (Hagberg, Gressens et al. 2012; Liu and McCullough 2013). Under physiological conditions the blood brain barrier excludes circulating immune cells and therefore the brain is considered an immunoprivileged site (Bucky Jones, Lucin et al. 2007). However, the brain possesses other immunological mechanisms to protect itself against harm, where both resident microglia and astrocytic cells have the capacity to elicit immune responses (McRae, Gilland et al. 1995).

5.1.1 Inflammation after brain injury

5.1.1.1 Cell death triggers immune-responses

Hypoxic ischemia causes an imbalance between energy consumption and production when oxygen and nutrient supplies are insufficient. If energy failure is severe enough, brain cells cannot handle the stress and die. The modes of cell death can theoretically be divided into controlled apoptosis and unrestrained necrosis (Thornton, Rousset et al. 2012). Apoptosis is an orchestrated energy dependent process that can be induced both intrinsically due to DNA damage or cell stress, and extrinsically due to extracellular death signals, causing negligible effects on extracellular environment (Thornton, Rousset et al. 2012). In contrast, necrosis causes disruption of cell membrane integrity and leakage of intracellular constituents (Northington, Chavez-Valdez et al. 2011) that function as danger associated molecular patterns (DAMP)/Alarmins to trigger immune responses (Bianchi 2007). In reality, a continuum exists between apoptosis and necrosis and an ample diversity of mechanisms mediating cell death (Northington, Chavez-Valdez et al. 2011).

5.1.1.2 Brain resident immune responses

The first immunological response to HI brain injury is activation of resident brain cells like microglia (Denker, Ji et al. 2007) and astrocytes (Pekny and Nilsson 2005). Microglial cells can directly cause increased secondary injury due to TNF-α induced neurotoxicity (Kaushal and Schlichter 2008). Noxious nitric oxide release has also been implicated. Microglia act as antigen presenting cells (APCs) through upregulation of

HLA molecules, co-stimulation and cytokine production (Lai and Todd 2006), thus providing all three signals necessary for T-lymphocyte activation (Kapsenberg 2003). However, microglia are not only harmful since they are involved in clearance of cellular debris and produce beneficial growth factors important for regeneration and recovery after a brain lesion.

Astrocytes are crucial for regulation of extracellular glutamate, an excitatory amino acid neurotransmitter that can cause excitotoxic lesions if abundant (Alvarez-Diaz, Hilario et al. 2007). Moreover, astrocytes have a major impact on blood brain barrier function and play a key role in regulating both blood flow and blood brain barrier permeability (Dare, Schulte et al. 2007). Astrocytes display some features coherent with antigen presenting capacity, however, if it actually occurs is controversial.

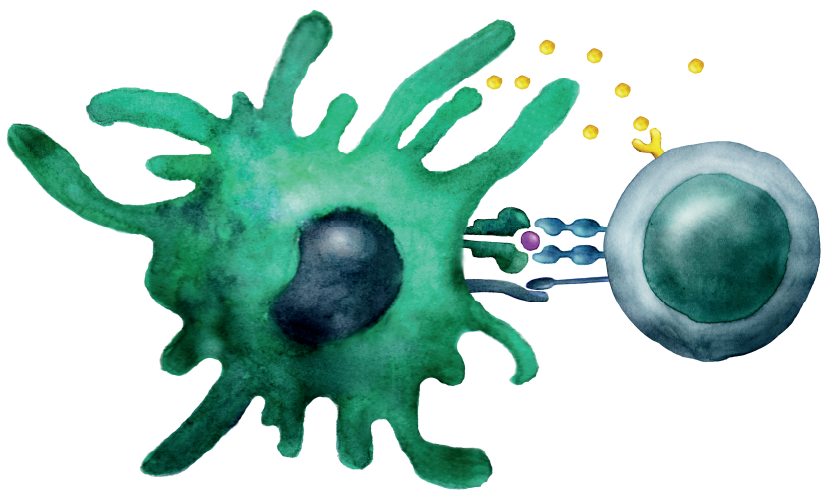

Figure 2 To elicit a specific T-lymphocyte mediated immune response, an antigen presenting cell (APC) (green) present the antigen (plum) mounted on the major histocompatibility complex (MHC) to the T-lymphocyte (blue). The MHC-peptide complex is recognized by the T-cell receptor providing signal one in T-lymphocyte activation. Co-stimulation necessary for activation is provided through signal two; CD80 or CD86 expressed on antigen presenting cells and recognized by T-lymphocytic CD28. Signal three consists of cytokines (yellow) released by the antigen presenting cell to direct the type of T-lymphocyte response.

5.1.1.3 Infiltration of systemic immune cells

The hypoxic ischemia induced breakdown of blood brain barrier function opens the brain for infiltration of systemic immune cells (Bona, Andersson et al. 1999; Hedtjärn, Mallard et al. 2004).

Innate immunity

Macrophages are one of the first cell types to infiltrate the brain after hypoxic ischemia, responding to DAMPs and cytokines (Denker, Ji et al. 2007). Phenotypically infiltrating macrophages and brain resident microglia are virtually indistinguishable, and thus the distinction between them functionally is an academic question. However, their anatomical location and temporal distribution in relation to the infarction matters, since cells separated on these criteria express different markers and have thus slightly alternative functions (Perego, Fumagalli et al. 2011). Microglia/macrophage activation is probably harmful since blockade of inflammatory mediators produced by these cells

is protective (Perego, Fumagalli et al. 2011). However, since it is impossible to completely deplete or block microglia/macrophage cells in brain there is no indisputable evidence. Two alternative ways of activation has been described, pro-inflammatory M1 and the regulatory anti-inflammatory M2 response (Lawrence and Natoli 2011). M2 induced macrophages seem to protect against hypoxic ischemia *in vitro* but not *in vivo* (Desestret, Riou et al. 2013), possibly because local environment *in vivo* is more important in microglia/macrophage function than intrinsic activation in this study. Nonetheless, understanding of microglial function and regulation continues to be an area of great interest for the development of neuroprotective strategies.

Natural killer cells (NK-cells) are involved in, as the name implies, cytotoxic killing and seem to play a role in neonatal brain injury (Bona, Andersson et al. 1999; Fathali, Ostrowski et al. 2013). However, their contribution to brain injury and mechanistic function during brain injury is not well described.

Neutrophils infiltrate the adult brain after brain injury, but their influence in the neonatal brain appears to be minor (Liu and McCullough 2013).

Adaptive immunity

T-lymphocyte activation after neonatal HI brain injury has been demonstrated indirectly (Bona, Andersson et al. 1999; Hedtjärn, Mallard et al. 2004). Classical activation of T-lymphocytes requires antigen presentation by antigen presenting cells (APCs) to naive T-lymphocytes. Co-stimulatory signals are necessary for the T-lymphocyte activation, and soluble and membrane bound factors directs the type of response launched (Kapsenberg 2003). With regards to brain injury, T- and B-lymphocytes depleted adult animals were protected as early as 22h after middle cerebral artery occlusion (MCAO) suggesting other acute functions than initiation of specific antigen recognition with subsequent clonal expansion and proliferation. Blockade of lymphocyte trafficking also protected the adult brain (Liesz, Zhou et al. 2011). Furthermore, corresponding to the situation in microglia/macrophages there is also a T-regulatory anti-inflammatory population that have been reported to convey neuroprotection (Liesz, Suri-Payer et al. 2009). Although these data suggest that lymphocytes are detrimental after ischemic brain injury, the functional status in the neonatal setting and whether they are able to respond to brain antigens is unknown.

B-lymphocytes are best known for their ability to produce antibodies, but they are also able to present antigens to T-cells (Ciechomska, Lennard et al. 2011). The existence of regulatory B-lymphocytes (B10) has also recently been proposed (Mauri and Bosma 2012). However, the regulatory function appears to be a transient state involved in normal activation and not necessarily a discrete function (Maseda, Smith et al. 2012), which seems reasonable since it would be beneficial to limit unspecific activation once a specific response to the presumed threat is present. Nonetheless, B-cell deficient mice develops larger infarction after MCAO in the adults (Offner and Hurn 2012). Offner *et al* suggest adoptive transfer of regulatory B-lymphocytes as a therapeutic approach for neuroprotection. However, this (or any transfer of immune populations with capability of immunological memory) could potentially be hazardous with risk of immunological reactivation later in life and induction of autoimmunity (Hagberg, Gressens et al. 2012).

5.1.2 Preconditioning effects of systemic inflammation

Perinatal infections induce adverse cognitive development (van Vliet, de Kieviet et al. 2013), and cytokines (Aden, Favrais et al. 2010) or LPS (Hagberg, Peebles et al. 2002) treatments are used in experimental models to mimic and understand how this effect is mediated. While short term detrimental effects after brain injury are seen after IL-1, TNF-α and LPS treatment, protective properties are observed after somewhat longer time (Dammann and Leviton 1997; Mallard and Hagberg 2007; Kendall, Hristova et al. 2011). LPS, IL-1 and TNF-α share some of the intracellular signaling cascades explaining the similarity in effect (Ferlito, Romanenko et al. 2001). One way to explain the dual effect is the danger hypothesis proposed by Matzinger (Pradeu and Cooper 2012), stating that immune activation is directed towards harmful dangerous stimuli. Moderate doses of IL-1, TNF-α or LPS do not represent any genuine danger, and therefore the pro inflammatory deleterious response is abolished. This hypothesis could also be applied to the similarities between infectious inflammation and aseptic inflammation during brain injury. Since both presents as actual threats to cell survival, an immune response is launched to attempt to terminate the harm by cytotoxic killing of injured cells and clearing of extracellular debris.

5.1.3 TNF-α

TNF-α is produced by all major cell types in the brain, microglia, astrocytes and neurons, and is a key cytokine in immune responses. The effect of TNF-α signaling varies between brain regions, and has principal functions in brain physiology and homeostasis including effects on excitatory amino acids, long term potentiation and plasticity, implicating impact on memory formation (O'Connor 2013). Thus, during long term blockade of TNF-α, like in the treatment of juvenile idiopathic arthritis (Gerloni, Pontikaki et al. 2008), adverse effects on cerebral function occur. There are two receptors for TNF-α, receptor one (TNFR1) and two (TNFR2) with diverse (Sriram and O'Callaghan 2007) but somewhat overlapping effects. The receptors are involved in regulation of both apoptosis and proliferation via the TNF receptor associated death domain (TRADD) and pathways leading to subsequent activation of the transcript factors activator protein 1 (AP-1) and nuclear factor kappa beta (NFκB) (O'Connor 2013). However tentative, a connection with TNF-α to adenosine receptor signaling has also been implicated (Trincavelli, Tonazzini et al. 2008).

5.2 ADENOSINE

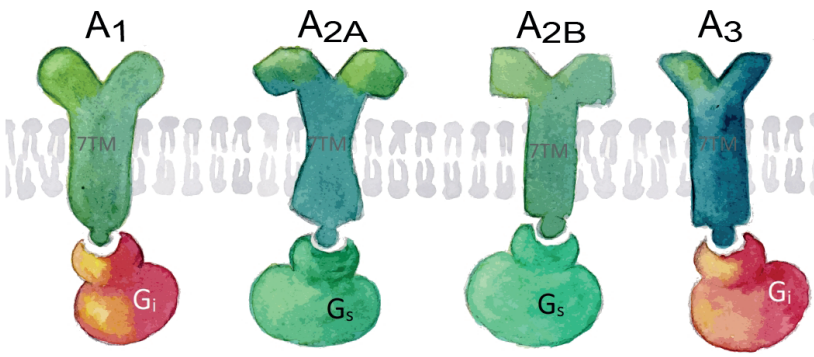

Figure 3 There are four subtypes of adenosine receptors A_1, A_{2A}, A_{2B} and A_3. All are coupled to G-proteins; A_1 and A_3 are coupled to G_i, inhibiting the formation of cAMP, whereas A_{2A} and A_{2B} stimulate cAMP through G_s coupling.

Adenosine signaling is situated at the crossroads of many fundamental functions involved in the homeostasis of physiological functions (Fredholm, AP et al. 2001). It is linked to the basic metabolism of every cell in the body since the majority of the available adenosine comes from degradation of adenosine triphosphate (ATP) a molecular transporter of chemical energy within the cell (Shampo, Kyle et al. 2011). It assists in the regulation of cardiac and vascular function, and adenosine administration is used clinically to stop and reboot the heart to treat heart arrhythmias. It is active in the brain where it regulates cell work, thus protecting the brain during hypoxic ischemia when energy is absent. Furthermore caffeine, the best known and most consumed adenosine modulating drug (limiting adenosine signaling) is often used for its stimulatory effect on brain in a variety of refreshing beverages. Furthermore, adenosine is involved in control of the breathing rhythm (Darnall and Bruce 1987) and body temperature (Fredholm, Johansson et al. 2011). Of particular interest in this thesis, adenosine also has major effects on immune regulation (Fredholm 2007; Linden and Cekic 2012). The above mentioned effects are only the ones of known importance in hypoxic ischemic brain injury. In addition, adenosine has known effects on skeletal muscles, kidney and fat metabolism (Fredholm, Johansson et al. 2011), and adenosine receptors are present in high levels in the eye, adrenal gland, ceacum, colon and in the bladder.

Still, it is possible to deplete mice of each of the four available adenosine receptors and the animals appear with essentially normal phenotype. This highlights that adenosine has a modulatory rather than controlling role during normal physiology. However during extreme physiology and pathology the impact is substantial (Fredholm 2007). During hypoxia adenosine signaling can exert both protective and detrimental effects. This functional diversity is achieved through differential intra cellular signaling pathways in combination with dynamic regulation of receptor distribution and density. There are four subtypes of adenosine receptors A_1, A_{2A}, A_{2B} and A_3, where A_1 and A_3 are coupled to G_i that inhibit the formation of cAMP while A_{2A} and A_{2B} receptors are coupled to G_s, and thus stimulate cAMP formation (Figure 3). Moreover, the receptor subtypes induces various other intracellular signaling pathways, thus further differentiating the signal (Fredholm, AP et al. 2001).

The affinities for adenosine vary between the receptor subtypes, where A_1 has the highest and A_{2A} and A_3 lower binding in decreasing order. A_{2B} has the lowest binding affinity and hence is only activated during extreme physiology or pathology like hypoxic ischemia (Fredholm, AP et al. 2011).
During brain hypoxic ischemia, adenosine levels available for signaling are mainly from extracellular breakdown of ATP. Disruption of the cell membrane during hypoxic ischemic injury leads to leakage of abundant intracellular ATP, readily cleaved by the ectonucleotidases CD39 and CD73 to form adenosine. During pathology, the extracellular concentrations can increase four to five orders of magnitude. Adenosine is degraded by adenosine deaminase (ADA), which thus acts to limit exaggerated adenosine levels.

5.2.1 Adenosine as an immune modulator

Adenosine has a profound impact on immune function and deletion of the enzyme responsible for degradation of adenosine, adenosine deaminase, results in severe combined immunodeficiency (SCID) and altered immune cell function (Cassani, Mirolo et al. 2008). Extensive research has been attributed adenosinergic immuno-modulation and most immune cell populations express some or all of the four adenosine receptor subtypes, depending on functional status and activity (Fredholm 2007; Hoskin, Mader et al. 2008; Kumar and Sharma 2009). In the context of tumor immunology a substantial accumulation of knowledge has been made regarding adenosine mediated immune regulation both in immune compartments and among tumor infiltrating cells (Kumar 2013). However, after brain hypoxic ischemia with a dissimilar microenvironment, much less is known and age differences further restrain unambiguous extrapolation from the present data obtained from tumor and *in vitro* experiments in adults.

5.3 CAFFEINE

Caffeine is currently used as a treatment for apnea of prematurity and in a randomized clinical trial been found to decrease the incidence of cerebral palsy and cognitive delay at eighteen months (Schmidt, Roberts et al. 2007). Furthermore, MRI findings report improved white matter development after caffeine treatment in preterm children (Doyle, Cheong et al. 2010). Nonetheless, the use of caffeine has been hampered due to safety concerns regarding; neurotoxicity, impaired development, and detrimental effects on long term behavior. Caffeine as a treatment in neonates was first proposed 1973 by Kuzemko *et al* as a treatment for apneas*,* although the effect on respiration was described as early as 1927 by Vogl (Lucey 1975). The Cap study 2006-2007 potentially expanded the indications for caffeine use to include neuroprotection (Schmidt, Roberts et al. 2007).
Caffeine is a competitive inhibitor of all adenosine receptors except the adenosine A_3 receptor that has low affinity for caffeine (Fredholm, Battig et al. 1999). Since A_{2B} receptors are only activated when adenosine levels are high (see above) the effect of caffeine in physiological conditions is mainly due to A_1 and A_{2A} effects, although A_{2B} probably plays a role during hypoxic ischemia. Furthermore, caffeine has the potential to inhibit the enzyme phosphodiesterase (PDE). Many of the (adverse) effects from caffeine have been attributed to PDE inhibition. However, phosphodiesterase inhibition requires 20 times higher concentrations than its effects on adenosine receptors (Fredholm, Battig et al. 1999), well above the concentrations reached during treatment.

The inhibition of PDE after therapeutic doses in neonates is probably minor (Aden 2011). Caffeine in clinically relevant concentrations could also potentially increase adenosine deaminase activity after brain hypoxic ischemia when adenosine levels are high (Leon, Michienzi et al. 2007; Xu and Venton 2010), thus further limiting excessive adenosine signaling.

5.3.1 Adverse effects of caffeine

Impaired growth in neonates has been reported in studies of suboptimal design after maternal caffeine intake, reviewed by (Aden 2011), however the only randomized double-blind controlled trial found no effects on birth weight (Bech, Obel et al. 2007). It is problematic to assess effects of caffeine in human studies, since intake is exceedingly difficult to estimate and the effect confounded by a number of factors *e.g.* smoking, alcohol, race and education (Valero De Bernabe, Soriano et al. 2004). Furthermore, it has been suggested that cytochrome P450 1A2 function, the enzyme responsible for caffeine degradation and clearance, is correlated to growth retardation rather than caffeine itself, that merely acts as a pseudo marker for cytochrome p450 1A2 function (Grosso, Triche et al. 2006).

Behavioral changes have been reported in rodents after prenatal caffeine exposure, however, prospective human studies concluded that the long term consequences at the age of seven was null (Barr and Streissguth 1991; American Psychological Association. Division of General Psychology. 1997). Postnatal high dose caffeine (20 mg/kg/day compared to the normally used dose of 5 mg/kg/day) given to preterm infants as apnea prevention found no adverse outcomes (Steer, Flenady et al. 2003; Steer, Flenady et al. 2004; Gray, Flenady et al. 2011) at one and two years of age.

In conclusion, there is no evidence in unbiased high quality studies of persistent detrimental effects in neonates when clinically relevant therapeutic concentrations of caffeine are used. Due to the intricate nature of caffeine intake and biological function, only randomized, controlled, prospective studies are appropriate to consider when attempting assessing adverse outcome without significant bias.

6 AIMS

In this work we aimed to study the mechanisms of hypoxic ischemic brain injury in the neonatal period with focus on inflammation and adenosine receptor signaling as targets for neuroprotective intervention.

1. To study if blockade of TNF-α offers protection against brain injury in two different models of immature brain injury
2. To investigate immune cell activation locally in the brain and systemically in spleen after brain hypoxic ischemia.
    - ❖ To develop a flow cytometry based method of investigation
    - ❖ To describe adaptive immune activation.
3. To assess the effect of adenosine A_1 receptor deletion on outcome after hypoxic ischemic brain injury.
    - ❖ To adopt data driven analysis for brain infiltrating immune cells and statistically discriminate genotype and injury size effects.
    - ❖ To investigate if brain inflammation, could be a significant factor affecting outcome after brain hypoxic ischemia in adenosine A_1 receptor deficient neonatal mice.
4. To evaluate the effect of adenosine A_{2A} receptor deletion on brain injury and post ischemic inflammation
5. To test if a single dose of 5 mg/kg caffeine after brain hypoxic ischemia is neuroprotective and the effect on cellular immune activations.

7 MATERIAL AND METHODS

7.1 HYPOXIC ISCHEMIA

In mice pups unilateral elecrocoagulation of the carotid artery was performed under Isoflourane sedation and local Bupivacaine anesthetic. Extensive care was taken to avoid stress to the mothers that when herself was calm, seemed able to calm the pups after the procedure.
Each animal operation typically takes less than 3 minutes per animal. Since longer isoflourane exposure could interfere with outcome (McAuliffe, Loepke et al. 2009), only animals operated within one hour were used in the same experimental run. After one hour of recovery and feeding with the dam the pups were put in hypoxia at 10% oxygen for one hour, to induce unilateral focal brain lesions similar to those seen in hypoxic ischemic encephalopathy of newborn children (Vannucci 2004). We have used ten day old mice that are similar to newborns in respect of white and grey matter development (Clancy, Finlay et al. 2007). There is however important differences between mice and men. Firstly, mice lack sulci, an important feature of the human brain cortex. They also have a different behavioral repertoire; they are nocturnal, small in size and depend on other primary senses than humans. Smell is the primary sense of mice and thus the olfactory bulb of mice is substantially larger and the major input wiring of neurons is thus somewhat different than in man. Having said that, the similarities are extensive and 99% of the mouse genome has direct counterparts in man (Gunter and Dhand 2002), which is why the mouse is considered an appropriate model to study brain injury. Even if an observation in the mouse not necessarily translates directly to the same function in humans, there is a great likelihood that similar molecular mechanisms can be found and studied. Mice studies also offer a great opportunity, as it is possible to eliminate genes of interest to test their function in different experimental models. There are *in vitro* models available but when studying immune cell responses after brain injury, an intact immune system, a blood brain barrier and vascular circulation is required.

7.2 EXCITOTOXIC BRAIN INJURY

Ibotenate an NMDA and metabotrobic glutamate receptor agonist was injected intracranially to induce excitotoxic brain lesions, where the major deleterious effect was mediated through NMDA activation (Marret, Mukendi et al. 1995). We used five day old mice, an age comparable to week 24-30 in humans a period during brain development when immature oligodendrocytes are exceedingly vulnerable to injury, causing lesions similar to periventricular leukomalacia of preterm babies (Hagberg, Peebles et al. 2002).

7.3 BEHAVIORAL EVALUATION

Functional deficits is the impairment that is perceived after brain injury, not infarct size or any other change seen in the brain, thus we investigated the behavioral effect of hypoxic ischemic brain injury. We used a small array of tests, always according to a detailed preset protocol and in the same order with the operator blinded to the variable investigated. Since, mice are easily disturbed and react to the operators behavior, smell and handling the experiment protocol included all handling of litters and mice, even

before the actual experiment started, and a recently showered, unparfumed and peaceful operator, to ensure a calm and explorative state of the mice. First we used the open field test where the movement and behavioral patterns is recorded with sensors and/or a camera in an empty acrylic glass box. A quiet, undisturbed surrounding is crucial since every sound directly will influence the behavioral reading. To minimize the bias from unexpected disturbances, cases and controls were run simultaneously, thus they should be comparable although noise is added to the behavioral signal. Directly after open field test we performed the beam walking test, where the mice balance a one centimeter wide beam three times, while slips with the hind limbs are counted. This test was the most sensitive detecting brain injury in our hands and simultaneously the most sensitive for disturbances. When stressed mice run over the beam in an attempt to escape, they perform fairly well even with brain injury. However, when calm they start to investigate the beam that triggers an explorative behavior rendering inattention of motor performance and unveiling functional deficits. A test less vulnerable to stress, is the Rotarod test, where the mice attempts to stick to an accelerating rod and time on or maximum speed is recorded. In this purely motor function test a certain activity and excitement in the mouse boost performance. To avoid misinterpretation, fear or pain is not excitement and the mice should appear enriched when performing the test.

7.4 IMMUNOHISTOCHEMISTRY

The brains collected for immunohistochemistry were first percoronary perfused with PBS to avoid autofluorescence from vascular erythrocytes. The brains were collected, snap frozen on dry ice and then cryosectioned in 10-14 µm slices. The tissue was fixated in 4% Paraformaldehyde for 10 min and blocked with serum from the same host as the secondary antibody or horse serum 3-50% volume concentration. We have found that higher serum concentrations up to 50% reduced background more efficiently, however most antibodies used in this work had a sufficient noise to signal ratio why there was no need for such high serum concentrations. Primary antibody was used after titration of optimal concentration and with irrelevant primary antibody as negative control. Secondary antibodies were either species specific, isotype specific or mouse on mouse kit was used according to manufacturer's specifications. All secondary antibodies were tested without primary antibody to assess unspecific binding. Detergent (triton-x100, saponin or Tween-20) were used in the blocking solution for intracellular staining (Jamur and Oliver 2010) and also for the last wash to break hydrophobic nonselective binding of antibodies. For light microscopy, horseradish peroxidase staining was typically performed and preceded by blocking of endogenous peroxidase activity with 0.3% hydrogen peroxide. Please see manuscripts for detailed protocols used in each study. To evaluate density of the staining and to be able to compare antigen expression between treatments or genotypes, the enzymatic step was performed simultaneously for all samples, ensuring comparability and integrated density was measured (pixels stained times intensity of staining in each pixel).

7.5 ELISA

Cells lysis buffer was used to disrupt cell membranes in the presence of phenylmethyl sulfonyl fluoride (PMSF) to avoid protein degradation. The lysate was centrifuged and supernatant collected for cytokine analysis using commercial ELISA kits in accordance to the manufacturer´s specifications.

7.6 WESTERN BLOT

Cells were lysed and proteins were separated through consecutive centrifugation steps. Protein concentrations were measured and a fixed amount of protein was denaturated with Laemmli sample loading buffer at 95°C. To further disrupt secondary, tertiary and quaternary structures natriumlaurilsulfate (SDS) was used and proteines separated using polyacrylamid gel electrophoresis and electrotransferred to a nitrocellulose membrane. Reversible Ponceau S staining was used to confirm equal loading and protein transfer. Proteins of interest were immunostained and detected by the enhanced chemiluminescence system (ECL, Amersham) and densiometrically analyzed with the image analysis environment TINA.

7.7 PROLIFERATION ASSAY

Thymidine incorporation based proliferation assays with brain homogenate as stimulus were set up to investigate the proliferation of spleenocytes from animals subjected to hypoxic ischemic brain injury and sham operated controls. The time to peak proliferation was estimated and used as optimal evaluation time point for each antigen prior final experiments, for details see paper II.

7.8 REAL TIME QUANTITATIVE POLYMERASE CHAIN REACTION

Messenger ribonucleic acid (mRNA) was extracted from brain tissue adjacent to the injury site by two consecutive total RNA isolation procedures including a DNAse I digestion step to degrade contaminating genomic DNA. Reverse transcription was performed using Iscript kit from Biorad. Sample not exposed to reverse transcription was used as negative controls to exclude remaining contamination of genomic DNA. All experiments were run in duplicates. Primers were designed using Oligo 6.0 and M-fold software. For primer sequences see manuscript I.

7.9 FLOW CYTOMETRY

The brains were perfused percoronary with 10 ml PBS to flush out circulating blood cells, since we set out to investigate brain infiltrating immune cells and not the vascular compartment. The cerebellum and the frontal part of the brains were removed and the infarcted and contralateral hemispheres were collected so as to include the part of the brain where the infarction is, and contralateral side. Tissues were then put in ice cold medium and kept cool to avoid in vitro activation and cell death. The brains were homogenized using a loose fit glass homogenizer and filtered through a 100 μm filter to remove supportive tissue and large cells that could clog the cytometer. Erythrocytes were lysed with hypotonic ACK buffer for five minutes, which preferentially removes cells without nucleus that are more sensitive to the hypotonic condition. No additional purification steps were performed to avoid in vitro activation and manipulation (Fung, Esposito et al. 2010). Other groups sometimes use Percoll purification (Gelderblom, Leypoldt et al. 2009). We have found that every extra cleaning step preferentially drains the activated compartment of tissue infiltrating cells (unpublished data), that induces bias. Thereafter cells were stained with fluorescence conjugated antibodies, washed and analyzed in the flow cytometer.

7.10 DATA DRIVEN ANALYSIS OF FLOW CYTOMETRY DATA

We deployed a R based approach of probabilistic data driven analysis using the flowCore (Hahne, LeMeur et al. 2009) and flowViz (Sarkar, Le Meur et al. 2008) packages that are reported to perform well (Aghaeepour, Finak et al. 2013). We adapted the methodology to brain infiltrating populations since the presentation of immune cells in solid tissue differs substantially from the roundish form in liquid where guidelines for gating exist (Calvelli, Denny et al. 1993). We chose to use a probabilistic function to interpret the populations in brain since the populations have not been previously investigated and normal distribution could not be assumed. Since the brain in an unchallenged state is fairly immuno privileged (Bucky Jones, Lucin et al. 2007) and often no clear positive immune cell population could be found, we localized the negative population and cells with higher expression of the marker investigated were assumed to be positive. All gates were manually confirmed for appropriateness, but after fine tuning of the algorithm no further adjustments were needed and the same settings were used in all experiments investigating the same markers in the same locations, *i.e.* brain and spleen respectively.

7.11 STATISTICAL ANALYSIS

For comparison of two experimental groups t-test was used when normal distribution could be assumed, otherwise the non-parametric Mann-Whitney test of pairwise comparison was used. Chi-square test was used to detect the frequency distribution of mortality. ANOVA multiple comparisons and Neuman-Keuls, Tukey or Dunnett post hoc test when appropriate to analyze multiple factors influencing the outcome of a variable. General linear model (GLM) regression was used to evaluate correlations between variables. Furthermore, we used principal component analysis (PCA) to reduce the dimensionality of multiplex flow cytometry data, allowing us to analyze and separate genotype effects from the effects due to brain injury size in our model. T^2 Hotelling and squared prediction error was used to validate the integrity of the data sets. The Nonlinear Iterative Partial Least Squares (NIPALS) algorithm was selected since it allows for inclusion of categorical values (like genotype) and thus allowed us to evaluate the covariation of genotype and the immune populations investigated. To group immune populations that had a similar representation on the components identified in the PCA analysis, and thus may have similar or connected functions we used expectation maximization clustering that uses an probabilistic approach to assign cluster belonging. To identify the appropriate number of clusters to analyze we used v-fold cross validation to avoid the potential bias that occurs when cluster number is assigned subjectively. Thus we minimized the need for extensive experiments and maximized the amount biologically relevant information extracted from the dataset without introducing major bias from brute force (analyzing all possible combinations of variables) large scale data analysis.

8 RESULTS AND DISCUSSION

8.1 TNF IN NEONATAL BRAIN INJURY (PAPER I)

We found that systemic IL-1β induced inflammation increased the susceptibility to brain injury in the preterm excitotoxic model. This effect is abolished through pharmacological blockade of TNF-α (Etanercept) given after the hypoxic ischemic insult or deletion of the TNF gene, producing a lesion size equivalent to those in animals not subjected to the systemic IL-1β. IL-1β treatment increased TNF-α production that thus seemed to mediate the detrimental effect. However, we performed a pilot study to investigate IL-1β induced systemic inflammation in a full term model of hypoxic ischemia and preliminary data suggests a preconditioning protective effect of five day IL-1β treatment (unpublished data). Thus age and mode of injury appear crucial for outcome, and the premature period might be associated with increased susceptibility to excitotoxic injury during inflammation.

We then continued to investigate TNF blockade in a more naturalistic inflammatory insult by injecting systemic lipopolysaccharide (LPS), a lipoglycan expressed on gram negative bacteria, 24 hours prior the hypoxic ischemic insult in the full term model. We found no significant differences between Etanercept pre- or post-treated animals compared to PBS treated controls (Figure 4). Since TNF blockade seems to protect only against inflammatory sensitization (Aden, Favrais et al. 2010) and it is now known that LPS induced sensitivity to hypoxic ischemia is greatest twelve hours after systemic administration (Kendall, Hristova et al. 2011) and even induce preconditioning protective effect 24 hours after administration (Mallard and Hagberg 2007), our findings are in retrospect, not surprising. Nonetheless, it stresses the importance of timing in a possible TNF targeted neuroprotective treatment. In combination with beneficial TNF effects (see background) as growth factor and regulator of neural function there are major hurdles to overcome to allow clinical use, and still only a fraction of the children (those with systemic inflammation) would potentially benefit from the intervention, why TNF blockade is not a primary candidate for neonatal neuroprotection today.

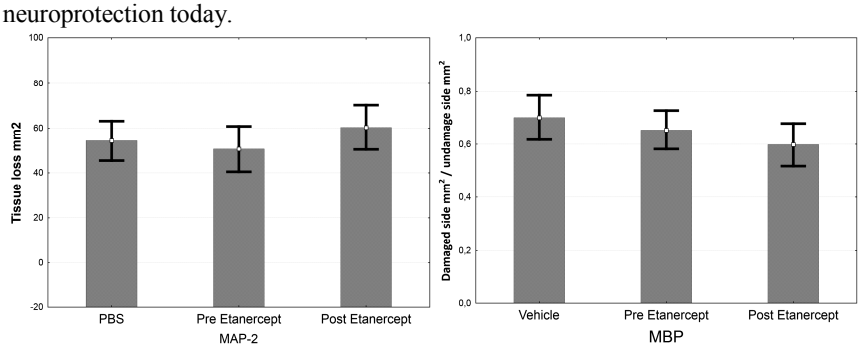

Figure 4 There were no significant differences due to TNF blockade with the decoy receptor Etanercept given before or after hypoxic ischemia, neither in total tissue loss seen by loss of MAP-2 staining nor in myelin content visualized by myelin basic protein (MBP).

8.2 BRAIN IMMUNE-INFILTRATION AND SYSTEMIC ACTIVATION (PAPER II)

The presence of adaptive immune cells in the brain after neonatal brain injury has been described (Bona, Andersson et al. 1999; Hedtjärn, Mallard et al. 2004). However, the temporal correlation between key immune populations and their interplay was unknown. We set out to investigate the adaptive component of immune activation and the relationship with innate immune activation and antigen presentation locally in the brain and systemically in the spleen. Since the number of adaptive immune cells was expected to be low compared to other cell types in the brain and we sought to investigate functional markers involved in activation and initiation of an adaptive immune response, we developed a multiplex flow cytometry based methodology to investigate both brain parenchyma infiltrating and systemic immune activation in the spleen after brain hypoxic ischemia.

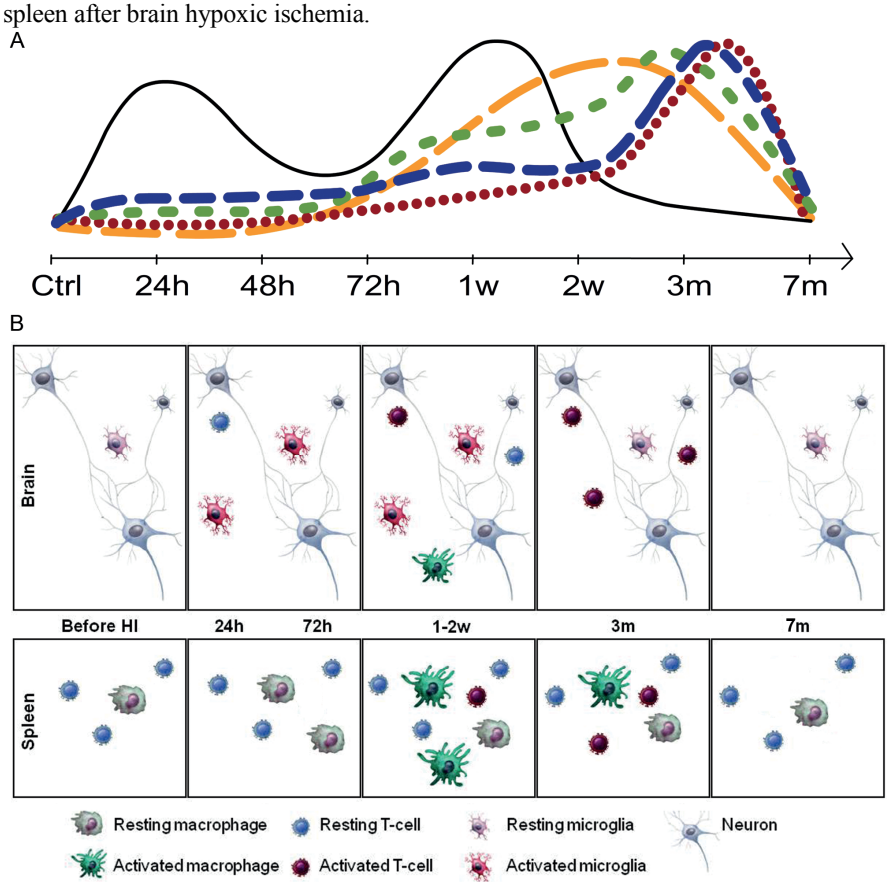

Figure 5 Cellular immune profile in brain and spleen after neonatal hypoxic ischemia. 24 hours and two weeks after injury $CD11b^{+}CD86^{+}$ cells increase in a biphasic pattern (black line) coherent with initial activation of brain resident microglia followed by infiltration of systemic macrophages. Very early activation antigen $CD69^{+}$ T-lymphocytes were found in the spleen (red dotted line) on week after the lesion, preceding $CD8^{+}$ cytotoxic T-lymphocyte activation and brain intrusion (Green short dashed line). T-lymphocyte activation continued at least three months, when the highest number activated $CD8^{+}$ cytotoxic and $CD4^{+}$ helper (blue long dashed line) were found, returning to pre insult levels seven months after brain hypoxic ischemia.

Innate immune activation was observed 24 hours after brain hypoxic ischemia and a second peak one week after the lesion. The first peak has been reported to consist of brain resident cells while the second peak represents infiltrating systemic immune cells (Denker, Ji et al. 2007). Furthermore, we could visualize an increase in the fraction innate cells expressing the costimulatory molecule CD86 in the spleen one week after the lesion, suggesting systemic antigen presentation. Interestingly, inhibition of this activation through spleenectomy has now been reported to ameliorate the infarction volume after neonatal hypoxic ischemia (Fathali, Ostrowski et al. 2013). Subsequent activation of antigen presenting cells, preferentially activated $CD8^+$ cytotoxic cells, was found in the brain and spleen at the same time, one week after the lesion. At this time a large proportion were naive T-lymphocytes expressing CD45rb, indicating that they had not yet encountered their cognate antigen. Intriguingly IL-4 and IL-13 production with suggested anti-inflammatory properties have been described after hypoxic ischemia (Shrivastava, Llovera et al. 2013). However IL-4 and IL-13 are able, at least under some conditions, to activate and promote proliferation and memory phenotype in $CD8^+$ cytotoxic T-lymphocytes (Morris, Heidorn et al. 2009), hence their function seem more coherent with a transition towards adaptive immune activation. The highest T-lymphocyte activation was observed in the brain three months after the lesion, then consisting of $CD4^+$ T-helper and $CD8^+$ cytotoxic cells, both of mainly $CD45rb^-$ effector phenotype. This phenotype was confirmed by the fact that spleenocytes from animals subjected to brain hypoxic ischemia lost their tolerance toward central nervous system antigens and proliferated when stimulated with brain or spinal cord homogenate. The ability of T-lymphocytes to respond to brain antigens persisted unaltered for at least five months once developed. Thus T-lymphocyte activation strongly suggests a long term effect of T-lymphocytes in the pathology after brain injury and possibly long term development of autoimmunity upon reactivation due to cellular damage in brain initiated by, for example infection or trauma, that could result in persistent immune mediated neuropsychiatric disability (Hagberg, Gressens et al. 2012). Interestingly, a recent publication suggested that mesenchymal stem cells transplantation protected the brain after global hypoxic ischemia partly due to diminished T-lymphocyte infiltration and induction of tolerance (Jellema, Wolfs et al. 2013).

8.3 ADENOSINE A_1 DEFICIENCY (PAPER III)

Adenosine A_1 receptor deficient mice were more susceptible to brain hypoxic ischemia than wild type mice. Increased injury in the A_1 deficient mice manifested as functional deficits in behavioral tests and as increased infarction size in histological sections.

One major hurdle to overcome when using multiplex flow cytometry is to reproducibly produce gate settings to select the immune cell populations for analysis. This is particularly difficult in brain parenchyma where immune populations acquire a more ramified structure and somewhat altered cellular distribution of the antigens investigated (as seen in immunohistological sections). We therefore developed a probabilistic approach to gate assignment that excluded the manual bias and allowed exactly the same result to be produced when reanalyzed, and consistent addition of new data when included. Furthermore, as this approach used the density of the populations and we used a simple binary strategy determining positive or negative cells, thus fluctuations in the laser intensity due to intrinsic variability in the flow cytometer could be accounted for. In order to validate the selection of populations to some degree, we

investigated the distributions and found that the data driven gating strategy generally produced populations more coherent with normal distribution. Since this finding also could be explained by the algorithm producing nonsense populations that inherently followed normal distribution we manually confirmed all gates in all samples and experiments and found the gates to be assigned satisfactory.

After analyzing the data one population at a time using ANOVA to investigate sham or hypoxic ischemic treatment, and wild type and adenosine A_1 deficient genotype the overall picture was still elusive. It was difficult to assess which populations were altered in a similar way and thus might have similar or connected function. We therefore deployed principal component analysis (PCA) using the nonlinear iterative partial least squares (NIPALS) algorithm that allows inclusion of dichotomous data such as genotype in our case. We were not able to experimentally assess damage size in the same animals that were used for flow cytometric analysis due to technical limitations. However, we realized that we could use principal component analysis to discriminate between genotype and injury size since the injury induced variance was similar in both genotypes. Principal component analysis construct components consisting of the combined covariation in the variables investigated thus reducing the dimensionality, where every additional component is by definition uncorrelated to the previous one. We extracted two components including only the significantly affected immune populations which could describe as much as 90% of the variance in our model. When plotting genotype against sham operated or hypoxic ischemic brain injury we could identify immune populations affected by the lesion size only, by genotype or a combination of the two. Furthermore, when each animal was plotted against the components they formed individual clusters. Thus we hypothesized that it should be possible to genotype the animals using the immune profile in sham and after brain injury. In fact we were able to correctly classify both treatment received and genotype in 90% of the cases using expectation maximization clustering and v-fold cross validation to determine correct number of clusters. Interestingly, some alterations in the immune responses were better explained by genotype than lesion size where the most profound were diminished activation of cytotoxic T-lymphocytes and regulatory IL-10 producing B-lymphocytes. The absence of a cytotoxic response in the A_1 deficient mice could reflect a shift in cell death mechanism from apoptosis to necrosis since adenosine A_1 receptors are known to protect against excitotoxicity during the insult (Fredholm 2007) – manifested as increased infarction size after hypoxic ischemia. The altered response in B-lymphocytes that produced IL-10 implies impaired B-regulatory cell function (Mauri and Bosma 2012). Furthermore, we found decreased overall levels of IL-10 that at least to some extent could explain the observed phenotype and suggests a novel mechanism of adenosine A_1 receptor mediated control of B-regulatory cells (B10) in tissue.

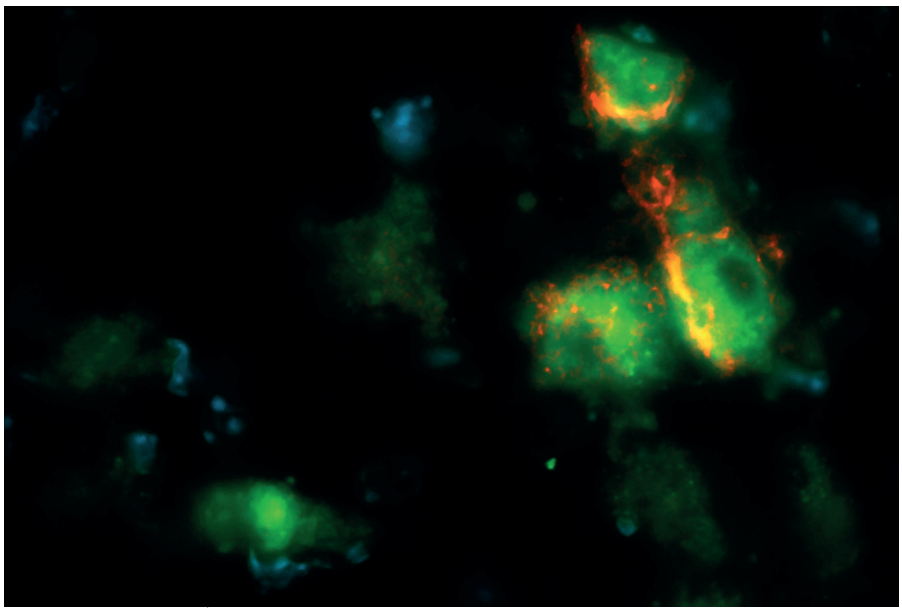

Figure 6 Most brain $B220^+$ B-lymphocytes (red) produced IL-10 (green), the hallmark cytokine of B-regulatory cells (B10) two weeks after hypoxic ischemia. Not all IL-10 producing were B-lymphocytes.

8.4 ADENOSINE A_{2A} DEFICIENCY (PAPER IV)

Both protective and detrimental effects of adenosine A_{2A} receptor blockade have been reported after hypoxic ischemic brain injury. In this paper we present a novel hypothesis that short term (hours to days) blockade is beneficial through non-immunological mechanisms and long term (two weeks) detrimental, likely through immuno-modulatory effects as suggested by our data (see below). The most prominent effects in the adenosine A_{2A} receptor knockouts after brain hypoxic ischemia were in regulatory anti-inflammatory populations like myeloid derived suppressor cells that were significantly fewer in the knockout. Adenosine is known to mediate a substantial part of the T-regulatory anti-inflammatory effect (Sitkovsky, Lukashev et al. 2009), and accordingly there was a substantial compensatory increase in $CD4^+FoxP3^+$ T-regulatory cells after brain injury in A_{2A} knockout mice. Deficiency in immune regulatory functions in adenosine A_{2A} knockout mice was paralleled by increased activation in cytotoxic natural killer cells and $CD8^+$ T-lymphocytes. Moreover, $CD86^+CD11b^+$ microglia/macrophages were significantly increased, coherent with the larger injury seen in the knockouts compared to wild type. Since short term adenosine A_{2A} receptor blockade is protective and long term detrimental timing of blocking strategies appears essential for outcome. The alteration in immune activation seen in this paper suggests a detrimental function of adenosine A_{2A} receptor deletion and supports the hypothesis that the delayed injury after neonatal hypoxic ischemia is caused by immune activation.

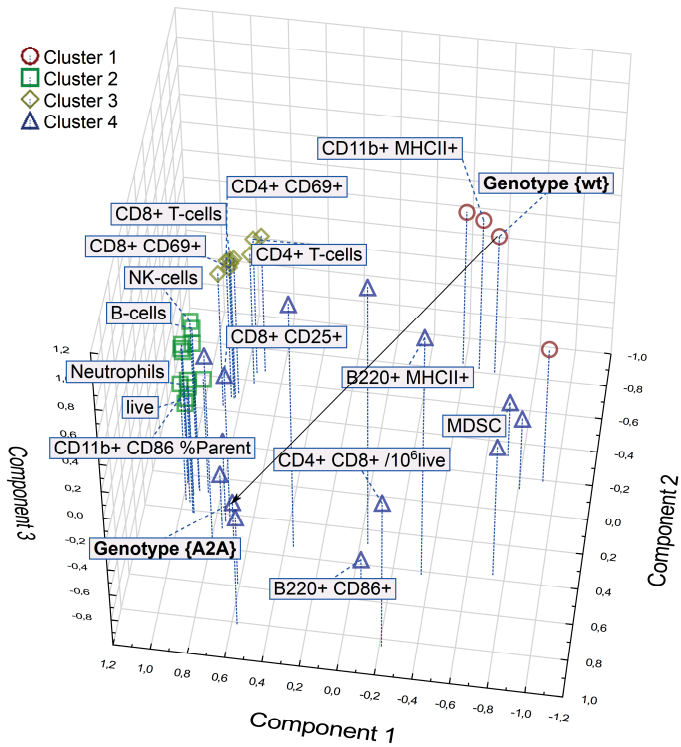

Figure 7 3D representations of key immune populations plotted against the three principal components acquired by the Nonlinear Iterative Partial Least Squares (NIPALS) algorithm, enabling inclusion of dichotomous data like genotype (connected by the arrow). The genotype effect is largely expressed on component 2. Component 1 effectively separates populations with protective (leftmost) or detrimental (to the right) effects according to prior knowledge reported elsewhere. Since we know that lesion size will contribute to the variance in a fashion that is uncorrelated to genotype and that the three components together explain 91% of the total variance in the model, injury size must thus be represented on component 1 or component 3. Since we found that the adenosine A_{2A} deficient mice influence injury size, genotype should be represented on the component describing lesion size, therefore we can exclude component 3 with no loading of genotype. The immune populations that influence component 3 (blue dashed lines) positively is regulatory B-lymphocytes and myeloid derived suppressor cells expressing the major histocompatability complex class II and negatively influenced by costimulatory ($CD86^+$) B-lymphocytes. However, since component 3, explaining 11% of the variance, was not influenced by adenosine A_{2A} receptors, it was excluded from the manuscript. V-fold cross validation with expectation maximization clustering was used to group the populations. The cluster containing adenosine A_{2A} deficiency (blue triangles) included regulatory immune populations, and the wild type (red circles) contained $CD11b^+$ $MHCII^+$. Proinflammatory innate populations were found tightly grouped in one cluster (green squares) while helper and cytotoxic T-lymphocytes were found in another cluster (yellow diamond).

8.5 CAFFEINE FOR NEUROPROTECTION (PAPER IV)

To investigate short term inhibition of adenosine receptor signaling we investigated a single dose caffeine 5 mg/kg given directly after hypoxic ischemia and found protection of the brain and improved behavioral outcome. We hypothesized that caffeine may modulate the inflammatory response after brain injury; yet, the alterations observed were minimal with transiently decreased number of activated $CD8^+$ cytotoxic T-lymphocytes, similar to the situation in adenosine A_1 receptor deficient mice. However, this population was only significantly different to controls after 24 hours and no significant difference was seen after 72 hours or two weeks. Thus, an immuno-modulatory effect was only seen when sufficiently high caffeine concentrations could

be expected. The protective effect of a single dose of caffeine was coherent with the results seen after short term adenosine A_{2A} receptor blockade in other studies. Long term caffeine treatment of the mothers prior hypoxic ischemic brain injury is another possible administration route of caffeine for neuroprotection (Bona, Aden et al. 1995). When caffeine is used for apneas, repeated doses of caffeine are given over time and treatment correlates with less neurodevelopmental disability (Schmidt, Roberts et al. 2007). This implicates that short term A_{2A} receptor blockade alone cannot explain the entire caffeine mediated effect and the balance between adenosine A_1, A_{2A} and A_{2B} receptors matters in the long run. Since electric activity and increased intracellular cAMP promotes neuronal survival (Goldberg and Barres 2000), there could hypothetically be a beneficial effect of long term caffeine due to central-nervous-system stimulation and modulation of cAMP levels. However, to our knowledge, long term caffeine treatment after neonatal brain injury has not been studied in respect to neuroprotection. Furthermore, caffeine treatment in combination with hypothermia, that is the treatment in clinical practice in the western world, has not been investigated. However, in settings where hypothermia is not available, caffeine appears, even with the current knowledge, as an appealing candidate for clinical neuroprotection.

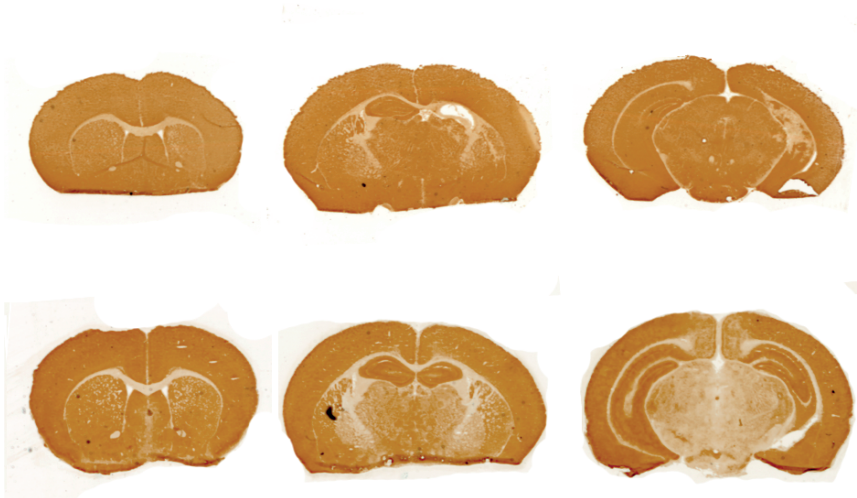

Figure 8 Loss of MAP-2 staining in neurons in three sections in the anterior-posterior direction delineates the infarction seen in buffered saline treated (top row) and after caffeine treatment (lower row). Each representative sample shown is from the brain with infarction size closest to mean in each treatment group. Especially the reduction of hippocampus lesions in the caffeine group (lower row) is clearly visible.

8.6 CONCLUSIONS

1. We investigated TNF blockade in the neonatal period and found that central blockade could protect the brain against excitotoxic lesions in premature pups during an inflammatory insult. However, no protection against brain hypoxic ischemia was seen without systemic inflammation or in mice at equivalent full term age. Thus we conclude that age and mode of brain injury are fundamental for the effect of TNF blockade for neuroprotection, and major obstacles have to be resolved prior to clinical application of such therapy.
2. Using flow cytometry we were able to demonstrate a long term immune infiltration in the brain parenchyma, months after neonatal hypoxic ischemia. Furthermore, splenocytes lost their tolerance against brain antigen after brain hypoxic ischemia and assumed a phenotype consistent with memory T-lymphocytes. Thus neonatal brain injury induces a long term immune response and the formation of an immunologic memory against brain antigens, with implications for development of autoimmunity and long term neurodevelopment.
3. Adenosine A_1 receptor deletion worsens the outcome after neonatal hypoxic ischemia and alters cerebral immune infiltration in profound ways. Mice deficient in adenosine A_1 receptors abolished the response in B-regulatory (B10) cells and cytotoxic T-lymphocytes seen in wild type mice after brain hypoxic ischemia. We discerned the genotype effect from lesion size elicited responses, and deduced that genotype caused specific alterations in immune activation. These alterations altogether correlated to increased brain injury in adenosine A_1 receptor deficient mice. We used principal component analysis to discriminate between two experimentally entangled but uncorrelated variables, thus this methodology could be used in experimental design to allow more than one deviating influencing factor.
4. Adenosine A_{2A} receptor deletion worsens the outcome after neonatal hypoxic ischemia and alters cerebral immune infiltration in profound ways. Functionally impaired myeloid derived suppressor cells and regulatory T-lymphocytes were seen in adenosine A_{2A} receptor deficient mice, paralleled by increased activation in cytotoxic natural killer cells and $CD8^+$ T-lymphocytes. Moreover, we present a novel hypothesis that short term adenosine A_{2A} receptor blockade offers neuroprotection while long term blockade is detrimental through immunological mechanisms.
5. Caffeine treatment given as a single dose 5 kg/kg is a clinically relevant neuroprotective strategy targeting adenosine signaling with few and manageable adverse outcomes. Are we ready for a clinical study?

8.7 CONCLUDING REMARKS

The technical evolution has given us access to more information and larger datasets. Thus, new demands on analysis and interpretation of the data have arisen. To prove a causational relationship in one factor is sometimes as misdirecting as a mere correlation (that is unable to prove causation), since nonlinear interactions and the synergistic effect of copious influencing factors with almost negligible individual effects often are disregarded. Computer based analytical approaches now seem matured to a sufficient level to guide us in big data analysis in biologically relevant ways. Mathematical or statistical coherent data is known to cause misinterpretation when not adapted to the biological reality. However, to acquire data of sufficient magnitude and quality is still a challenge.

Further utilization of principal component analysis on experimental data has powerful applications and could be used in clinical trials and epidemiology. Generally speaking, epidemiological approaches most commonly used perform well when large populations are available to analyze correlations between a limited numbers of variables. However large populations are not always available. Thus a similar approach as in paper three and four could be used to investigate few subjects but plenteous variables and to estimate biologically relevant interactions. For example investigating one outcome in 10000 subjects yields the same amount of information as 100 variables in 100 subjects. Correlation analysis handles the first situation excellently, while the latter situation with abundant, correlated variables will be problematic. Since principal component analysis does not require that the variables are uncorrelated, it is well suited for the latter problem. Furthermore, the identification of important variables with principal component analysis can be used to direct research efforts to a limited number of variables that experimentally could be exploited for causal relationships, for example by building Bayesian networks or performing a strict selection of key experiments.

These techniques seem appropriate and necessary to address the complexity of adenosine receptor signaling if one aims to understand and ultimately predict its function:

Adenosine signaling
Fundamental, although not required
Blocking can stimulate, and activation inhibit
Effective during extreme conditions, yet promotes normalization
Elicits local responses, yet is ubiquitously present
Elicits similar, and opposing effects
Affects body and mind

Great insight and knowledge can be gained by understanding its path.

9 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Syrebrist och otillräcklig blodförsörjning till hjärnan i nyföddhetsperioden är en allvarlig komplikation som kan ge men för livet. Målet med detta arbete har varit att förstå vissa aspekter av hur dessa skador uppstår och om möjligt hitta ett sätt att skydda hjärnan under denna avgörande period i livet. Den neonatala nyfödda hjärnan är normalt sett mindre känslig mot störningar i energiförsörjningen och syrebrist än den vuxna hjärnan, detta för att klara en förlossning. Om störningen blir så allvarlig att en hjärnskada uppstår kan dock även den pågående utvecklingen av den omogna hjärnan störas, och på så sätt orsaka dubbel skada. Samtidigt har den nyfödda hjärnan en stor kapacitet att kompensera för skador då den är plastisk och oskadade regioner av hjärnan kan ta över och utföra funktioner den vanligtvis inte har. Därför finns mycket att vinna på optimal behandling av asfyxiska barn med påverkad cirkulation och syresättning av hjärnan. Vidare har det visat sig att infektioner både kan starta för tidiga förlossningar och dessutom orsaka skador på hjärnans funktion. Idag är kylbehandling den enda specifika behandling for hjärnskador som finns tillgänglig för fullgångna barn, men för prematura, för tidigt födda barn, finns ingen specifik behandling alls. Stora framsteg i neonatalvården har ändå kunnat åstadkommas genom behandling av infektioner, införandet av steroider, symtomatisk akutvård samt uppföljnings- och stödinsatser under uppväxten.

I en musmodell av prematur hjärnskada med samtidig systemisk inflammation har vi visat att blockad av en inflammatorisk signalsubstans, cytokinen tumör nekros faktor (TNF) skyddar hjärnan. Detta skydd kunde bara ses vid samtidig inflammation. Då TNF förutom de immunologiska effekterna möjligtvis kan påverka både långtidsminne och hjärnanas utveckling och bara en andel av barnen potentiellt skulle ha någon nytta av behandlingen, de med systemisk inflammation, anser vi inte TNF blockad som neuroprotektion i nyföddhetsperioden är aktuell för klinisk applikation i en nära framtid.

Då infektioner har visats vara en riskfaktor för att utveckla hjärnskador vid syrebrist och denna effekt medieras av immunförsvaret, har vi utvecklat en metod för att med flödescytometri undersöka immuncellers förmåga att infiltrera hjärnan i en neonatal musmodell, motsvarande den nyfödda mänskliga hjärnan. I den modellen har vi även kunnat testa olika tänkbara behandlingsregimer för att utvärdera dess potentiella nytta.

Immuncellernas funktion är att skydda oss mot infektioner av bakterier, virus och parasiter, men de rensar även bort skadade och döende celler. Detta är normalt en bra funktion som skyddar mot tumörutveckling och påskyndar läkning. Vid en hjärnskada kan svaret under vissa omständigheter bli alltför kraftigt, varför det kan uppstå ytterligare förvärrad skada istället. Vi har kunnat visa i vår musmodell att denna immunaktivering är en långvarig process som pågår i flera månader och inte veckor som varit känt sedan tidigare. Vi har även visat att denna immunaktivering ger upphov till ett immunologiskt minne, vilket innebär att de skadliga effekterna vid upprepad hjärnskada kan förväntas bli mycket värre. Man kan tro att denna aktivering kan ge upphov till autoimmunitet senare i livet och även påverka den neuronala utvecklingen då det finns beskrivet att ADHD, depression och autismspektrumstörningar är korrelerade till ökad immunaktivering.

Vi har även undersökt hur adenosinreceptorer påverkar hjärnskadan och immunaktivering. De flesta vuxna människor intar mer eller mindre regelbundet centralstimulerande substanser vars effekt medieras av adenosinreceptorer. Vanligen i form av uppiggande drycker såsom kaffe och te, men även som läsk och energidrycker, alla innehållandes koffein eller teofyllin som blockerar adenosinreceptorsignalering. Adenosin bildas från adenosin trifosfat (ATP), som är cellens energivaluta, varför funktionen är nära sammankopplat med cellernas energiomsättning, något som är uppenbart viktigt vid syrebristorsakade hjärnskador som ju orsakas av energibrist. När celler dör läcker ATP ut från cellens energiförråd och ombildas snabbt till adenosin som binder till och aktiverar någon av de fyra adenosin receptorer som finns på cellväggen. Då initieras en signalkaskad som påverkar cellens beteende och överlevnad. Vi har använt knockoutmöss som saknar en av de fyra adenosinreceptorerna, som vi sedan har jämfört med möss som har alla fyra receptorerna intakta och kunde då se att de möss som saknade en av adenosin receptorerna hade värre skador efter av syrebrist orsakade hjärnskador. Vi kunde även visa att avsaknad av adenosin A_1 eller A_{2A} receptorer har en stor specifik påverkan på immunaktiveringen efter en hjärnskada, något som tyder på att adenosinsignalering har en viktig funktion för ett lagom aggressivt immunsvar. De förändringar vi såg i knockoutmössen var korrelerade med värre skador, vilket vi tolkar som att störd adenosinmedierad immunreglering påverkar uppkomsten av skada.

Eftersom vi kunde se stora effekter i knockoutmössen och dessa påverkade utfallet, ville vi undersöka de terapeutiska möjligheter som fanns med modulering av adenosinsignalering, varför vi undersökte om koffein 5 mg/kg givet som en enkel dos efter syrebrist i hjärnan kunde skydda mot skada. Vid koffeinbehandling minskade skadorna och aktivering av cytotoxiska T-celler under pågående behandling sjönk också. Långtidseffekterna på immunaktivering efter en enda dos koffein var försumbara, vilket ger låg risk för långtidsbiverkningar, därmed har denna behandling uppenbar klinisk relevans. Biverkningsprofilen är också mycket fördelaktig när man ser till resultaten i väldesignade humana studier utan uppenbara felkällor. Koffein används dessutom redan i klinisk praxis som skydd mot andningsuppehåll hos för tidigt födda barn. Vi har dock inte studerat upprepade doser av koffein, vilket är vanligt i kliniken vid apnebehandling. Dessa resultat är ett steg emot en klinisk studie av koffein för neuroprotektion vid neonatal hjärnskada. Kanske är vi redo?

10 ACKNOWLEDGEMENTS

First, I want to thank all of you that have contributed with scientific insight and discussions that ultimately led to this thesis. To you that have helped me evolve through life. To you that contribute to knowledge and revelations to make the world a better place. Even if this includes all of you below I want to express my gratitude specifically to the following persons:

Min handledare Ulrika Ådén, som har handlett mig från den tid då jag var medicinstudent och nästan svimmade med händerna skakandes (3cm tremor) i närheten av en spruta (något som kanske borde fått dig att tvivla på min förmåga att genomföra mikrokirurgi på 4 grams möss). Tack för att jag har fått hållas när jag har haft vilda idéer, varav några faktiskt fungerat över förväntan. Du har under hela perioden bidragit med kliniska insikter, förutom de experimentella, som har hjälpt till att hålla fokus på det kliniskt relevanta, vilket ju varit syftet med denna avhandling.

Jag har genom livet haft nöjet att arbeta nära med erfarna genier inom sina respektive fält: Jacob Moscovicz, konsertpianist och min pianolärare, Ingmar Bergman som regisserade och Donya Feuer som var koreograf under min tid som elev på Dramaten, och nu Bertil Fredholm, farmakolog som trots att han inte officiellt varit min handledare lagt ner exceptionellt mycket tid och bidragit med sitt extensiva förstånd, kunnande och insikter, samt agerat som riktig vetenskaplig förebild.

Min bihandledare Ola Winqvist, som har en otrolig förmåga att entusiasmera och hitta framkomliga vägar i besvärliga situationer. Som är så medryckande när du berättar om forskningsframsteg på obotliga sjukdommar, att man på ett ogenomtänkt sätt önskar att man hade sjukdomen, bara lite. Dessutom bestitter du en djup immunologisk kunskap, som varit helt avgörande för utformningen av denna avhandling. Du har dessutom alltid ställt upp och snitsigt kommit på lösningar när arbetet verkligen kört fast, även om du befunnit dig på andra sidan jordklotet.

Min medförfattare, labbkompis, vetenskapliga bollplank, klagomur, musa, räddare i nöden, som skapat akvarellerna i denna bok, fru samt moder till mina underbara barn. Tack Malin Winerdal för att du står ut med mig!

All my co-authors, for your dedication and contribution to scientific knowledge.

Ett stort tack till de som delat med sig av sina kunskaper och färdigheter som på ett konkret sätt bidragit till utformningen av denna avhandling och/eller för mig rent personligen bidragit till min vetenskapliga utveckling, i blindad randomiserad ordning: Ruth Detloffson, Gunnar Schulte, Per Marits, Jonas Berner, Eva Lindgren, Karin Lindström, Tomas Ringstedt, Lilian Sundberg, Anders Arner.

Jag tackar för det oförglömliga minnet av att övernatta i ljungen utanför Göteborgs Universitet då nyckeln till mitt rum var inlåst för natten och alla hotell var fullbokade. Viktigare, vill jag även tacka Catherine Rousset och Carina Mallard som lärde mig vannuccimodellens hemligheter.

Tack till dig som jag bara sett en enda gång och vars namn jag tyvärr inte la på minnet men som tillfälligt dök upp som en räddande ängel i labbet när jag förtvivlat försökte hitta lymfknutor utifrån en bok. Du tog dig tid och lärde mig hitta och plocka ut lymfknutor jag inte ens visste fanns på ett effektivt sätt.

Till Erik Carlson, min mentor under avhandlingstiden som bidragit med både praktiska insikter och tänkvärda anekdoter och till din fantastiska familj Angelica, Tilde och Ingrid samt bror Anders.

Tack till kollegor, vänner för samvaro och intressanta disskussioner rörande både högt och lågt: Klas Blomgren, Hugo Lagerkrantz, Erik Herlenius, Emilija Wilson, Lena Swartling, Lea Forsman, Emma Lindh, Emilie Grasset, Evelina Lindmark, Ludvig Linton, Ali Zirakzadeh, Emma Ahlén-Bergman, Lars Björk, Olga Björklund, David Krantz, Eva Lundberg, Jenifer Frithiof, alla trevliga medarbetare på ALB samt L2:04 samt dig jag glömt att nämna vid namn.

Julian Rex, min "partner in crime" när vi började utforska forskningens komplexa värld.

Alla vänner från den medicinska grundutbildningen, särskilt Alexandra Grahn, Pearl Edlin, Ulrika Thorö, Robert Larsen, Karin Sundström och Beatrice Kvist: Tack för alla roliga stunder.

En särskild tanke till Per och Carina Carlson.

Ett tack till dig som inspirerade mig till att frälsas av naturvetenskapen och börja studera på komvux och följt mig genom livets alla skeden, min bror Jens Winerdal.

Till mina föräldrar Lars och Elisabet Winerdal: Ok då, nåt måste ni väl ha gjort rätt...

Till mina svärföräldar Mats Blennow och Elisabeth Syk Lundberg, svågrar Mattias Blennow och Victor Blennow, samt Ana Rodriguez-Sanchez och alla andra medlemmar från svärfamiljen för allt ifrån trevligt familjeumgänge till veteskapliga diskussioner.

The production babies: Mina helt underbara, fantastiska och enastående barn, Mira och Matilda.

Och slutligen till Otis: Du förstår mig i alla fall...

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